Mechanism-based Predictions of Interactions

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Exposure to more than one toxic compound is common in real life. The resulting toxic effects are often more than the simple sum of the effects of the individual compounds. It is unlikely that it will ever be possible to test all combinations. It is therefore highly desirable to improve or develop means for reasonably approximating predictions of interactions. In order to be valid and extrapolatable, these predictions are most promising if they are mechanism-based. Examples will be given for possibilities of mechanism-based predictions of interactions which exceed trivialities of simple increases by enzyme induction of enzymatic rates of a given biotransformation pathway leading to a toxic metabolite. Instead, examples will be provided where competition between various enzymes for shunting the same substrate into divergent pathways can lead to predictable dramatic changes in toxicity by shifting the metabolic routes under conditions of no significant changes of overall metabolism. Further examples are given on predictable interactions between chemicals which need bioactivation for exerting their toxicity and chemicals which effect hormonal status and other endogenous factors which in turn modify enzymes involved in the control of toxic metabolites.—Environ Health Perspect 102 (Suppl 9):5–9 (1994)

Key words: drug–drug interactions, interactions between foreign compounds (xenobiotics), protein phosphorylation, enzyme induction, enzyme inhibition, genotoxicity

Introduction

Interactions between xenobiotics based on enzyme inhibition can be very profound. Figure 1 shows an example of inhibition of the major microsomal epoxide hydrolase mEH_b* at low concentration (0.3 µM) of 1,1,1-trichloropropene 2,3-oxide (*2*). At this concentration, no influences are known on any other enzyme contributing to the control of epoxides by either forming them or metabolizing them further. In the absence of this, modulator phenanthrene does not induce mutations in *Salmonella typhimurium TA*1537 to a measurable extent in agreement with the known noncarcinogenicity of the compound. However, 0.3 µM trichloropropene oxide leads to a pronounced mutagenicity. This concentration of the modulator led to an inhibition of mEH_b (measured with the standard substrate benzo[*a*]pyrene 4,5-oxide which has a similar affinity to the enzyme as phenanthrene 9,10-oxide) of approximately 95%, that is to a lowering of the effective mEH_b activity by a factor of roughly 20. Phylogenetic investigations had shown that the mEH_b activities varied between individual vertebrate species by up to about 1000-fold (*3*). Thus, modulations by inhibitors far lower than variations in mEH_b activities occurring between vertebrate species were sufficient to convert an apparent nonmutagen to a clearcut mutagen by sufficiently raising the steady state concentration of the responsible epoxide.

*mEH_b: The major microsomal epoxide hydrolase possessing a broad substrate specificity diagnostically including benzo[*a*]pyrene 4.5-oxide (*1*).

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Interactions between Xenobiotics Based on Enzyme Induction

The well known mutagenic carcinogen benzo[*a*]pyrene is virtually nonmutagenic when activated by liver microsomes from rats which had not been pretreated with

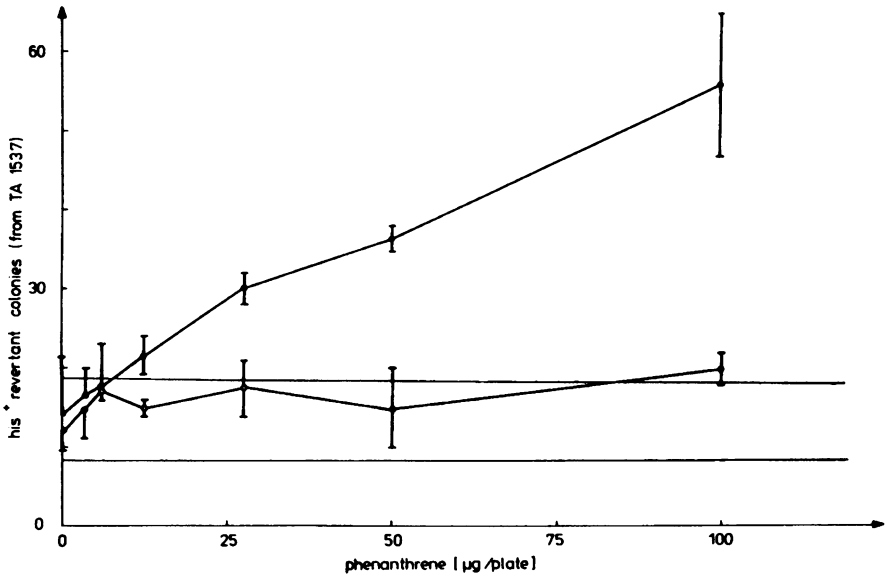

Figure 1. Dose dependency of the mutagenic effect of phenanthrene after activation with liver microsomes from mice induced with Aroclor 1254 (500 mg/kg) for *Salmonella typhimurium* TA1537. ○, incubation without addition of an inhibitor for epoxide hyratase. ●, 0,6 µl 1,1,1-trichloropropene 2,3-oxide in 10 µl dimethylsulfoxide was added to the incubation mixture. The horizontal lines indicate the range of numbers of colonies on plates without test compound ($n = 8$). Data from Buecker et al. (*2*); reproduced by permission of *Mutation Research*.

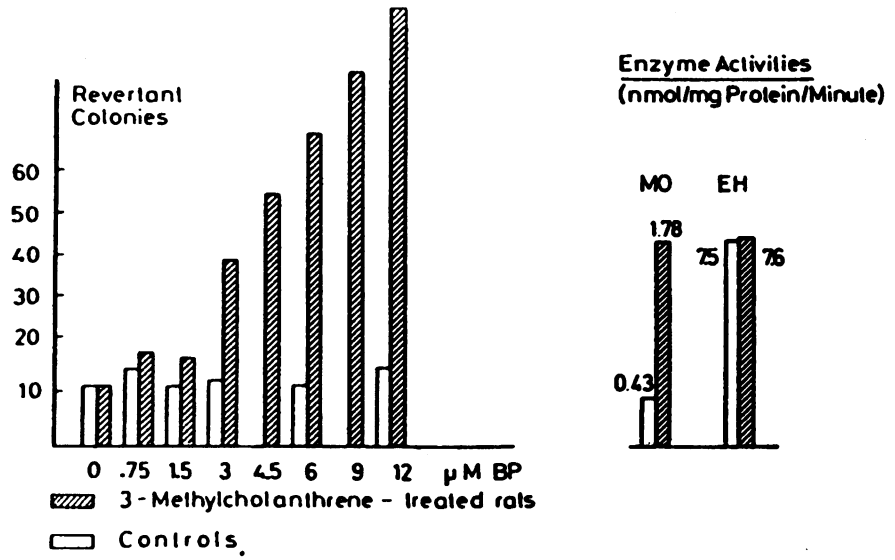

Figure 2. Activation of benzo[*a*]pyrene to a mutagen: potentiation by selective induction of monooxygenase. Benzo[*a*]pyrene and *Salmonella typhimurium* TA1537 bacteria were incubated with hepatic microsomes from control male Sprague-Dawley rats or from rats that had been pretreated intraperitoneally with 10 mg/kg of 3-methylcholanthrene in sunflower oil and killed 3 days later. Data from Oesch and Glatt (*4*); reproduced by permission of IARC publications.

enzyme inducers (*4*). However, high mutagenicity is observed after induction by 3-methylcholanthrene. The dramatic difference is shown in Figure 2. The special practical importance is that in some cases such enzyme inductions may be a prerequisite for the mutagenicity and carcinogenicity to occur. This may be through induction by a compound different from the compound in question (interaction) or by a dose of the compound which is sufficient for induction (potential threshold of genotoxicity and carcinogenicity due to induction of constitutively not induced enzymes).

Interactions between Xenobiotics Based on Shifts of Routes of Metabolism

We incubated [^{14}C]-benzo[*a*]pyrene with control and with trans-stilbene oxide (TSO)-induced rat liver microsomes and the metabolites were separated by HPLC (Table 1). Standard compounds and two different elution systems were used to characterize the metabolites. The results presented are derived from a separation using an acetonitrile-water gradient. Similar results were obtained using a methanol-water gradient with the exception that separation of the 4,5-epoxide peak from the 3,5-quinone peak was achieved with the acetonitrile-water but not with the methanol-water gradient. In addition, it was confirmed that metabolites,which were tentatively identified by their mobilities as dihydrodiols, were markedly reduced or disappeared when the inhibitor of mEH_b, 1,1,1-trichloropropene 2,3-oxide was present. As seen from Table 1, the total quantities of the metabolites was not significantly changed after TSO-treatment of the rats. However, a most remarkable shift of the metabolism occurred. The quantity of metabolites which were oxidized at the benzo ring (7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, 9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene, 9-hydroxybenzo[*a*]pyrene) was greatly diminished, while far more K-region metabolites (4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene, benzo[*a*]pyrene 4,5-oxide) were formed. The ratio between 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and 4,5-dihydroxy-4,5-dihydrobenzo[*a*]-pyrene was more than 20 times lower with TSO-induced microsomes than with control microsomes. Only insignificant changes were observed in the peaks containing the quinones and 3-hydroxybenzo[*a*]pyrene. As expected from the induction of mEH_b by TSO, a much higher percentage of the metabolically pro-

Table 1. Effect of *trans*-stilbene oxide administration on the pattern of benzo[*a*]pyrene metabolism by rat liver microsomes.[a]

Radioactivity with mobility of the following reference compounds	Control rats		Trans-stilbene oxide treated rats		Ratio[b] TSO-treated/control
	pmoles, mg protein × 20 min	% of metabolites	pmoles, mg protein × 20 min	% of metabolites	
9,10-Dihydroxy-9,10-dihydrobenzo[*a*]pyrene	1240 ± 90	13.5	460 ± 10	4.3	0.37
4,5-Dihydroxy-4,5-dihydrobenzo[*a*]pyrene	430 ± 10	4.7	2670 ± 250	25.0	6.21
7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene	330 ± 10	3.6	90 ± 30	0.8	0.27
Benzo[*a*]pyrene 1,6-quinone and 3,6-quinone	3690 ± 20	40.2	3750 ± 480	35.0	1.01
Benzo[*a*]pyrene 4,5-oxide	430 ± 90	4.7	760 ± 70	7.1	1.76
9-Hydroxybenzo[*a*]pyrene	320 ± 10	3.5	120 ± 10	1.1	0.37
3-Hydroxybenzo[*a*]pyrene	2190 ± 170	23.9	1840 ± 320	17.2	0.84
Others	550 ± 40	5.9	1030 ± 80	9.5	1.87
Total	9180 ± 390	100.0	10700 ± 1250	100.0	1.16

[a] Data from Buecker et al. (*5*); reproduced by permission of *Molecular Pharmacology*. [b] Ratio of metabolites present after 20 min incubation with microsomes from *trans*-stilbene oxide treated and control rats.

duced benzo[*a*]pyrene 4,5-oxide was converted to the corresponding dihydrodiol with TSO-induced microsomes.

Various doses of benzo[*a*]pyrene were activated by microsomes or postmitochondrial fractions to mutagens which were detected by the reversion of various *his*⁻ *Salmonella typhimurium* strains. In general, induction by TSO, markedly decreased the mutagenicity of benzo[*a*]-pyrene. The greatest reduction (by more than 90%) was obtained when post-mitochondrial fractions and the strain TA100 were used (Figure 3A). Using microsomes, TSO treatment reduced the mutagenicity with TA100 by 65% (Figure 3B) and with TA98 by 40% (Figure 3C, round symbols). However, TSO treatment only slightly reduced the mutagenicity with TA1537 (Figure 3D, round symbols). The differences can be easily explained. The *Salmonella* assay is a backward mutation assay which requires specific mutations to reconstruct a functional *his* gene. Different his⁻ strains vary in their susceptibility to reversion by different benzo[*a*]pyrene metabolites. TA100 and TA98 are easily reverted by benzo[*a*]pyrene 7,8-dihydrodiol 9,10-oxides, and by benzo[*a*]pyrene 4,5-oxide. The strain TA1537 is less sensitive towards the 7,8-dihydrodiol 9,10-oxides but is highly sensitive to the 4,5-oxide (*6*). The greater decrease of mutagenicity by TSO induction with the strains which are sensitive to 7,8-dihydrodiol 9,10-oxides (TA100, TA98) when compared with a strain which is relatively insensitive towards these dihydrodiol epoxides (TA1537), suggests that the reduction of the mutagenicity is caused to a significant extent, by the decreased oxidation of benzo[*a*]pyrene at the benzo ring. The decreased benzo ring metabolism is accompanied by increased K-region metabolism, leading to the mutagenic benzo[*a*]pyrene 4,5-oxide, but mEH_b, which is induced by TSO (*7*), can inactivate this mutagen (*4,8*).

The mEH_b inhibitor 1,1,1-trichloropropene oxide (*9*) potentiated the mutagenic effects both with control and with TSO-induced microsomes (Figure 3C,3D). This experiment could be performed only with TA98 and TA1537 since 1,1,1-trichloropropene oxide is too mutagenic with TA100 (*10*). With TA98, inhibition of mEH_b increased the mutagenicity of benzo[*a*]pyrene activated with control microsomes about 3-fold, and with TSO-induced microsomes about 5-fold (Figure 3C). Also, with TA1537, inhibition of mEH_b potentiated the mutagenicity more strongly when TSO-induced microsomes were used for the activation (Figure 3D). This shows the increased importance of mEH_b for the inactivation of mutagenic benzo[*a*]pyrene metabolites after a shift of the metabolism to the K-region, since the K-region epoxide benzo[*a*]pyrene 5,6-oxide is an excellent substrate of mEH_b, indeed is used as its diagnostic substrate (*1*).

Thus, shifts in routes of metabolism due to interactions between xenobiotics can profoundly change their biological effects.

Interactions between Xenobiotics Based on Posttranslational Modifications of Enzyme Proteins

Overall organismic activities of xenobiotic-metabolizing enzymes may change by altering enzyme amount or specific activity by induction, repression, activation, or inhibition. Very widely studied is the contribution of induction, which is a relatively slow process requiring the biosynthesis of the enzyme protein. The possibility of a faster regulation of xenobiotic metabolism by posttranslational modification (e.g., by phosphorylation) of an already preexisting protein has only recently received attention. A central role in the metabolism of xenobiotics is played by the cytochrome P450-dependent monooxygenases.

Purified cAMP-dependent protein kinase (PKA) and purified calcium/phospholipid-dependent protein kinase (PKC) catalyze, donor- and acceptor-selectively,

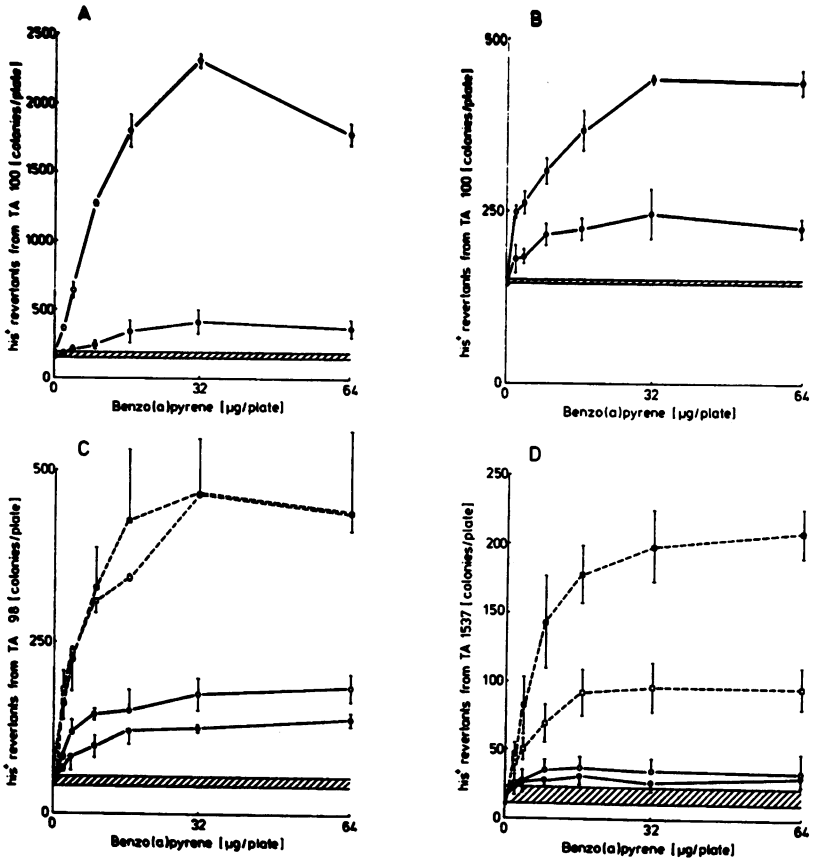

Figure 3. Effect of *trans*-stilbene oxide treatement on the activation of benzo[*a*]pyrene to mutagens. Male Sprague-Dawley rats (210–230 g) received intraperitoneal injection of *trans*-stilbene oxide (2 mmole/kg body weight, dissolved in 0.5 ml sunflower oil) or sunflower oil only 72, 48, and 24 hr before sacrifice. Various amounts of benzo[*a*]pyrene, 9000*g* supernatant fraction (A) or microsomes (B–D) of liver homogenate, a NADPH generating system and *his*⁻ *Salmonella* typhimurium (A,B: TA100; C: TA98; D: TA1537) were mixed with histidine-poor soft agar and poured on a minimal agar plate. In part of the experiments, 1 mmole of the mEH_b inhibitor 1,1,1-trichloropropene 2,3-oxide (*9*) was added. Colonies (*his*⁺ revertants) were counted after an incubation for 2 days at 37°C. The values represent means ± SD of three experiments with *trans*-stilbene oxide treated and of two experiments with control animals. Livers from two animals were pooled and two plates were run for each experimental condition. The hatched zones show means ± SD of the number of revertant colonies in the absence of benzo[*a*]pyrene. ○, control rats; ●, *trans*-stilbene oxide-treated rats. □, control rats with mEH_b inhibitor. ■, trans-stilbene oxide-treated rats, with mEH_b inhibitor. *A) S. typhimurium TA* 100,9000*g* supernantant. *B) S. typhimurium* TA 100, microsomes. C) *S.typhimurium* TA 98, microsomes. D) *S. typhimurium TA* 1537, microsomes. Data from Buecker et al. (*5*); reproduced by permission of *Molecular Pharmacology.*

the phosphorylation of some, but not all, purified cytochrome P450 isoenzymes, while the phosvitin/casein type II kinase was inactive for 13 cytochrome P450 isoenzymes investigated (*11–14*). In order to see whether this process also occurred in the intact cell, hepatocytes were exposed to ^{32}P-orthophosphate to label the intracellular ATP pools. Hepatocytes were isolated from the liver of adult male Sprague-Dawley rats pretreated with phenobarbital in order to induce the cytochrome P450 isoenzymes 2B1 and 2B2 (cytochromes P450b and P450e, the two major phenobarbital-inducible cytochromes P450). Among the 13 investigated isoenzymes, these two had been the best substrates of PKA in the cell free system (*12*). In the absence of stimulation, the incorporation of radioactive phosphate into cytochromes P450 (isolated and purified after the incubation.with 32P-orthophosphate) was very low. After stimulation by extracellular glucagon or by membrane permeating cAMP-derivatives (N^6,$O^{2'}$-dibutyryl-cAMP and 8-thiomethyl cAMP) a marked incorporation of ^{32}P-phosphate into cytochrome P450 took place (*15*). Autoradiography of gel electrophoretically separated proteins from solubilized microsomes of these hepatocytes and of purified cytochromes P450 combined with visualization on Western blots by specific antibodies showed that four cytochromes P450 were phosphorylated, cytochromes P450 2B1 and2B2, and two cytochromes P450 2B1-related proteins, one of them inducible by phenobarbital (*15*). Pyerin and Taniguchi (*16*) and Koch and Waxman (*17*) have also observed that the major phenobarbital-inducible cytochrome P450 is phosphorylated in the intact hepatocytes and in the whole animal (*17*) where phosphorylation took place in the liver in the absence of any exogenous stimulation and was increased by N^6, $O^{2'}$-dibutyryl-cAMP and theophyllin. Johansson et al. (*14*) reported on the phosphorylation of the ethanol-inducible cytochrome P450 isoenzyme 2E1 which was stimulated by cAMP analogues.

The phosphorylation of cytochromes P450 2B1 and 2B2 in intact hepatocytes led to a marked decrease in the microsomal monooxygenase activity towards substrates of these isoenzymes (*18*): The *O*-dealkylation of 7-pentoxyresorufin which represents a selective substrate of cytochromes P450 2B1 and 2B2 was markedly decreased; similarly, the metabolism of testosterone was markedly and regio- and stereo-selectively changed in those positions of the steroid molecule which are attacked by cytochromes P450 2B1 and 2B2. After treatment of the hepatocytes with the agents leading to phosphorylation of cytochromes 2B1 and 2B2 (cAMP derivatives), the 16β-hydroxylation of testosterone which is catalyzed by cytochromes P450 2B1 and 2B2 was decreased about 50% (*18*). The influence of the phosphorylation on the hydroxylation at the 16∝-position was "diluted" (decreased about 30%) by those isoenzymes which in addition to the phosphorylated cytochromes P450 2B1 and 2B2 also catalyze this reaction, namely cytochromes P450 2C7 (P450f, immunologically crossreactive with cytochrome P450 2Bl, constitutive isoenzyme), cytochrome P450 2C11 (P450h, male specific testosterone 2∝/16∝-hydroxylase) and cytochrome P450g (male specific, strain-dependent) (*19,20*). Cytochromes P450 2B1 and 2B2 also catalyze the oxidation of the 17β-OH group of testosterone to a keto group (conversion to androstenene-3,17-dione). However, this reaction is still less specific. Hence, the decrease in activity due to phosphorylation was even more "diluted" at this position and did not reach statistical significance. Jansson et al. (*21*) reported that in a cell-free system phosphorylation of cytochrome P450 LM2 (the major phenobarbital-inducible cytochrome P450 in the rabbit liver, P450 2B4), inhibited the interaction of the latter with the former, leading to a loss of the stimulatory activity of cytochrome b_5 prior to its reconstitution with cytochrome b_5. Taniguchi et al. (22) reported that in a cell-free system the treatment of cytochrome P450 purified from the liver of a phenobarbital-treated rabbit with purified PKA leads to a destruction of cytochrome P450 to cytochrome P420. Johansson et al. (*14*) reported that in hepatocytes the agents which lead to an increase of phosphorylation of cytochrome P450 2El (glucagon and cAMP analogues) also lead to an increase in the degradation rate of cytochrome P450 2E1. Agents which protected against the degradation also protected against phosphorylation. Based on these observations, the authors suggested that phosphorylation leads to a more rapid degradation of cytochrome P450 2E1. Thus, phosphorylation of cytochromes P450 may influence metabolism by modulating their activity as well as their levels.

The selective phosphorylation of defined cytochromes P450 by some defined protein kinases which themselves are under differential control in intact cells (and to the limited extent in which it has been investigated, also in animals) followed by highly selective and predictable changes in metabolic rates strongly suggests that interactions between xenobiotics may be expected on this level. Some expected interactions are listed in Figure 4. The issue gains further significance by our recent demonstration that these processes also occur in human tissue (Oesch-Bartlomowicz, Arens, Fähndrich, Vogel and Oesch, manuscript in preparation).

Figure 4. Interactions to be expected between compounds metabolized by cytochromes P450 phosphorylated by protein kinase A and compounds leading to increases in cAMP.

1. P450 2B1 substrates: Several cytostatic drugs (cyclophosphamide, ifosfamide), many barbiturates, several anticonvulsants.
2. P450 2E1 substrates: Many nitrosamines (dimethyl, diethyl, methyl,propyl, methyl, benzyl), vinyl halides, vinyl carbamate, urethan, acrylonitrile, styrene, benzene, many haloalkanes, chlorozoxazone.
3. Stimulators of adenylate cyclase: b-active sympathomimetics; endogenous stimulators released upon cachetic situation (glucagon) or upon cardiac insufficiency (adrenalin or noradrenalin, or both).
4. Compounds leading to decrease of adenylate cyclase activity: β-blockers.
5. Phosphodiesterase inhibitors: methylxanthines (theophylline, caffeine).

Conclusions

Interactions between xenobiotics may profoundly influence their toxic effects. The permutations of possible interactions are expected to lead to an infinite number of conceivable situations which may never be amenable to individual investigation. Development of possibilities for reasonable approximations and predictions are therefore desirable. They may be most effective if the underlying considerations are mechanism-based. This overview summarizes a few possibilities such as enzyme inhibition, enzyme induction, shift of routes of metabolism, and posttranslational modification of enzymes.

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